



Europäisches Patentamt  
European Patent Office  
Office européen des brevets

(11) Publication number:

**0 364 255  
A2**

(12)

## EUROPEAN PATENT APPLICATION

(21) Application number: 89310424.0

(51) Int. Cl.<sup>5</sup>: C12Q 1/68 , C07H 21/04

(22) Date of filing: 11.10.89

(30) Priority: 12.10.88 US 256689

(43) Date of publication of application:  
18.04.90 Bulletin 90/16

(84) Designated Contracting States:  
AT BE CH DE ES FR GB IT LI NL

(71) Applicant: BAYLOR COLLEGE OF MEDICINE  
One Baylor Plaza  
Houston, TX 77030(US)

(72) Inventor: Caskey, Charles T.  
6402 Belmont Street  
Houston Texas 77005(US)  
Inventor: Chamberlain, Jeffrey S.  
3117 Cason  
Houston Texas 77035(US)  
Inventor: Gibbs, Richard A.L.  
3602 Gramercy  
Houston Texas 77025(US)  
Inventor: Rainer, Joel, E.  
2100 Commonwealth R.  
Houston Texas 77006(US)  
Inventor: Nguyen, Phi Nga  
2300 Old Spanish Trail, Apt. 1032  
Houston Texas 77054(US)

(74) Representative: Adams, William Gordon et al  
RAWORTH, MOSS & COOK 36 Sydenham  
Road  
Croydon Surrey CR0 2EF(GB)

(54) Multiplex genomic DNA amplification for deletion detection.

(57) The present invention relates to a method for detecting multiple DNA sequences simultaneously. The method involves amplification of multiple sequences simultaneously by annealing a plurality of paired oligonucleotide primers to single stranded DNA. One member of each pair is complimentary to the sense strand of a sequences and the other member is complimentary to a different segment of the anti-sense strand of the same sequence. The amplification occurs by alternately annealing and extending the primers. The invention also includes oligonucleotide primer sequences helpful in detecting genetic diseases and/or exogenous DNA sequences.

EP 0 364 255 A2

## Multiplex Genomic DNA Amplification for Deletion Detection

### Field of the Invention

This invention relates to the field of simultaneous detection of deletions in genomic DNA sequences by the process of amplification of multiple sequences within the hemizygous or homozygous genome. The nucleic acid sequences are amplified by the process of simultaneous multiple repetitive reactions. This method of deletion detection is useful in a variety of areas including screening for genetic disease, and animal husbandry. Multiplex DNA amplification is also applicable to the simultaneous analysis of multiple genomic sequences and is useful in forensic medicine, disease screening, and in the development of recombinant or transgenic organisms.

### Background

This invention is an improvement on currently established procedures for the detection of genetic diseases resulting from mutations and deletions in genomic DNA sequences. Prenatal diagnosis and carrier detection of many X-linked diseases is available via Southern analysis using full length cDNA clones. Unfortunately, there are several major limitations that prevent widespread and routine use of Southern analysis for diagnosis of genetic disease. In many of the X-linked diseases, the defective sequences are unknown and probes are unavailable. In other diseases, such as X-linked muscular dystrophy, there are multiple exons, at least 60, scattered over a large area of genomic DNA, approximately 2.4 million bases. The introns average .35 Kb in length. In the case of muscular dystrophy, at least 7-9 separate cDNA subclones are necessary for Southern blot analysis to resolve each exon-containing restriction fragment for hypotype assignment or diagnosis of genomic alterations. Furthermore, Southern analysis is an expensive, tedious, and time-consuming technique that requires the use of radioisotopes, making it unsuitable for routine use in clinical laboratories.

An alternative to Southern analysis for mutation and deletion detection is the polymerase chain reaction (PCR) described by Mullis et al. in U. S. Patent No. 4,683,195 which issued on July 28, 1987 and by Mullis in U. S. Patent No. 4,683,202 which issued on July 28, 1987. With PCR, specific regions of a gene can be amplified up to a million-fold from nanogram quantities of genomic DNA. After amplification the nucleic acid sequences can be analyzed for the presence of mutant alleles either by direct DNA sequencing or by hybridization with allele-specific oligonucleotide probes. The PCR technique has proven useful in the diagnosis of several diseases including  $\beta$ -thalassemia, hemophilia A, sickle cell anemia and phenylketonuria. Routine screening for genetic diseases and exogenous DNA sequences, such as virus, with PCR, has been limited by the ability to conduct tests for only a single sequence at a time. Screening for a plurality of possible DNA sequences requires a cumbersome large number of separate assays, thus increasing the time, expense, and tedium of performing such assays. For example, in some diseases, such as Duchenne muscular dystrophy (DMD), PCR diagnosis has been limited since point mutations leading to DMD have not been identified. Approximately 60% of the cases of DMD are due to deletions. The other 40% are unknown at present, but probably involve mutations of the intron-exon splice sites or the creation of premature stop codons. Thus a large gene like the DMD gene must be screened with multiple assays.

In both U. S. Patent Nos. 4,683,195 and 4,683,202, procedures are described for amplification of specific sequences. Both patents describe procedures for detecting the presence or absence of at least one specific nucleic acid sequence in a sample containing a mixture of sequences. Although the patents claim at least one sequence and state that multiple sequences can be detected, they do not provide an effective procedure for amplifying multiple sequences at the same time. In the examples, single sequences are amplified or multiple sequences are amplified sequentially. Adding primers for a second sequence is usually possible, but when primers for more than two sequences are added the procedure falls apart. The present application is an improvement on the PCR method and solves the problems encountered when primers for multiple sequences are reacted simultaneously. The present invention describes a procedure for simultaneous amplification of multiple sequences, and the application of this multiplex amplification procedure to detect a plurality of deletions within the same gene or within multiple genes.

The procedures of the present application provide improved methods for the detection of deletions in hemizygous genes on the X and Y chromosomes. The procedures are effective in detecting genetic

diseases caused by deletions on the X or Y chromosome, for example, DMD. They are also effective in detecting homozygous deletions and may be used to simultaneously screen for many possible homozygous or hemizygous deletions as long as parts of the appropriate genetic sequences are known. The procedure for multiplex amplification also enables simultaneous analysis of multiple genetic loci regardless of the presence or absence of deletions.

### Summary of the Invention

10

An object of the present invention is a method for simultaneously detecting deletions at a plurality of genomic DNA sequences.

An additional object of the present invention is to detect X-linked genetic diseases.

A further object of the present invention is the diagnosis of DMD.

15

A further object of the present invention is to simultaneously analyze multiple genetic loci for polymorphisms and/or non-deletion mutations.

Thus, in accomplishing the foregoing objects there is provided in accordance with one aspect of the present invention, a method for simultaneously detecting deletions at a plurality of genomic DNA sequences, comprising the steps of:

20

Treating said genomic DNA to form single stranded complementary strands;

Adding a plurality of paired oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand;

25

Annealing the plurality of primers to their complementary sequences;

Simultaneously extending said plurality of annealed primers from each primer's 3' terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separating from their complement, serving as templates for the synthesis of an extension product from the other primer of each pair;

30

Separating said extension products from said templates to produce single-stranded molecules;

Amplifying said single stranded molecules by repeating at least once, said annealing, extending and separating steps; and

Identifying said amplified extension products from each different sequence.

35

Additional embodiments include detection of deletions at a plurality of genomic DNA sequences on the X and Y chromosomes or on autosomal chromosomes when the deletions are homozygous. A variety of X-linked diseases can be detected including ornithine transcarbamylase deficiency, hypoxanthine phosphoribosyltransferase deficiency, steroid sulfatase deficiency and X-linked muscular dystrophy.

In another embodiment, X-linked muscular dystrophy is detected using a plurality of paired primers which are complementary to different sequences within the gene coding for the protein dystrophin. Other

40

embodiments include multiple oligonucleotide primers useful in detecting X-linked genetic disease.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure when taken in conjunction with the accompanying drawings.

45

### Brief Discussion of the Drawings

The invention will be more readily understood from a reading of the following specification and by references to the accompanying drawings, forming a part thereof:

50

Figure 1 is a schematic representation of the DMD gene illustrating the approximate size of the locus, the position of the amplified fragments and the location of the genomic regions that have been cloned and sequenced.

55

Figure 2 is an example of a PCR reaction used to detect a deletion in fetal DNA for prenatal diagnosis.

Figure 3 represents the multiplex DNA amplification of lymphoblast DNA from unrelated male DMD patients. A. and B. show two sets of ten samples. Each DRL # refers to the R.J. Kleberg Center for Human Genetics Diagnostic Research Laboratory family number. MW: Hae III digested  $\phi$ X174 DNA. (-): no template

DNA added to the reaction. The relationship between the amplified region and the region on the gen is indicated to the right of A. The letters correspond to those on Figure 1.

Figure 4 represents Multiplex DNA amplification for prenatal diagnosis of DMD. Shown are the results of amplification using DNA from an affected male (AM; lymphoblast DNA) and a male fetus (MF; cultured amniotic fluid cell DNA) from six different families. Both the affected male and the fetal DNAs of DRL #s 521 and 531 display a deletion of region f (Fig. 1) diagnosing these fetuses as affected. In DRL # 43C the affected male is deleted for all regions except f, while the fetus is unaffected. The affected male in DRL # 483 is deleted for region a, while the male fetus is unaffected. Neither of the samples from DRL #s 485 or 469 displayed a deletion with this technique.

Figure 5 represents Multiplex DNA amplification from chorionic villus specimen (CVS) DNA. Both the affected male (AM; lymphoblast DNA) and the male fetus (MF; CVS DNA) from DRL # 92 display a deletion of regions e and f (Fig. 1), diagnosing the fetus as affected. CVS DNA from DRL # 120 did not display a deletion with this technique.

Figure 6 shows amplification of seven exon regions of the DMD locus.

The drawings are not necessarily to scale and certain features of the invention may be exaggerated in scale or shown in schematic form in the interests of clarity and conciseness.

#### Detailed Description

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein, without departing from the scope and spirit of the invention.

The term "oligonucleotide primers" as used herein defines a molecule comprised of more than three deoxyribonucleotides or ribonucleotides. Its exact length will depend on many factors relating to the ultimate function and use of the oligonucleotide primer, including temperature, source of the primer and use of the method. The oligonucleotide primer can occur naturally, as in a purified restriction digest, or be produced synthetically. The oligonucleotide primer is capable of acting as an initiation point for synthesis when placed under conditions which induce synthesis of a primer extension product complementary to a nucleic acid strand. The conditions can include the presence of nucleotides and an inducing agent such as a DNA polymerase at a suitable temperature and pH. In the preferred embodiment, the primer is a single-stranded oligodeoxyribonucleotide of sufficient length to prime the synthesis of an extension product from a specific sequence in the presence of an inducing agent. In the deletion detection procedure, the oligonucleotides are usually at least greater than 12 mers in length. In the preferred embodiment, the oligonucleotide primers are about 18 to 29 mers in length. Sensitivity and specificity of the oligonucleotide primers are determined by the primer length and uniqueness of sequence within a given sample of template DNA. Primers which are too short, for example, less than about 12 mers may show non-specific binding to a wide variety of sequences in the genomic DNA and thus are not very helpful. In the preferred embodiment, the oligonucleotide primer is usually selected for its ability to anneal to intron sequences in the proximity of the 5' or 3' end of the exon or to anneal to a sequence at the intron-exon junction. Since the known deletion defects resulting in genetic diseases result from deletions that include the exons or intron-splice site regions, it is preferable to have primers complementary to intron sequences.

Each primer pair herein was selected to be substantially complementary to the different strands of each specific sequence to be amplified. Thus, one primer of each pair is sufficiently complementary to hybridize with a part of the sequence in the sense strand and the other primer of each pair is sufficiently complementary to hybridize with a different part of the same sequence in the anti-sense strand. Thus, although the primer sequence need not reflect the exact sequence of the template, the more closely it does reflect the exact sequence the better the binding during the annealing stage.

Within a primer pair, each primer preferably binds at a site on the sequence of interest distant from the other primer. In the preferred embodiment the distance between the primers should be sufficient to allow the synthesis of an extension product between the two binding sites, yet close enough so that the extension product of each primer, when separated from its template, can serve as a template for the other primer. The extension products from the two paired primers are complementary to each other and can serve as templates for further synthesis. The further apart the binding sites, the more genomic DNA which can be screened. However, if the distance is too great the extension products will not efficiently overlap with the primers and thus amplification will not occur.

As used herein the term "extension product" refers to the nucleotide sequence which is synthesized from the 3' end of the oligonucleotide primer and which is complementary to the strand to which the

oligonucleotide primer is bound.

As used herein the term "differentially labeled" shall indicate that each extension product can be distinguished from all the others because it has a different label attached or is of a different size or binds to a specifically labelled oligonucleotide. One skilled in the art will recognize that a variety of labels are available. For example, these can include radioisotopes, fluorescers, chemilumescers, enzymes and antibodies. Various factors affect the choice of the label. These include the effect of the label on the rate of hybridization and binding of the primer to the DNA, the sensitivity of the label, the ease of making the labeled primer, probe or extension products, the ability to automate, available instrumentation, convenience and the like. For example, a different radioisotope could be used such as  $^{32}\text{P}$ ,  $^3\text{H}$ , or  $^{14}\text{C}$ ; a different fluorescer such as fluorescein, tetramethylrhodamine, Texas Red or 4-chloro-7-nitrobenzo-2-oxa-1-diazole (NBD); or a mixture of different labels such as radioisotopes, fluorescers and chemilumescers. Alternatively, the primers can be selected such that the amplified extension products for each sequence are of different lengths and thus can be separated by a variety of methods known in the art. Similarly, the extension products could include a restriction fragment length polymorphism which could be used to distinguish different extension products. In these examples, each primer or its extension product can be differentiated from all the other primers when they are in a mixture. Alternatively, probes which bind to the amplified extension products could be labeled and sets of probes which distinguish alleles of a single sequence within a multiplex DNA amplification reaction may be used whether or not labelled.

Each specific, different DNA sequence, which is to be detected herein, can derive from genomic DNA of the organism or exogenous DNA such as virus, bacteria or parasites. The source of genomic DNA from the organism to be tested can be blood, hair or tissue (including chorionic villi, amniotic cells, fibroblasts and biopsies). The source of DNA may be freshly obtained or have been suitably stored for extended periods of time. The DNA must be of sufficient quality to permit amplification. The genomic DNA can be prepared by a variety of techniques known to one skilled in the art.

As used herein, the term "deletion" refers to those genomic DNA sequences in which one or more nucleic acid base has been deleted from the sequence and thus is no longer present in the gene. The size of the deletion can affect the sensitivity of the amplification procedure. Generally, the larger the deletion the larger the sensitivity.

Any specific known nucleic acid sequence can be detected by the present method. Preferably, at least part of the sequence is deleted from the genome. It is only necessary that a sufficient number of bases at both ends of the sequence be known in sufficient detail to prepare oligonucleotide primers which will hybridize to the different strands of the desired sequence at relative positions along the sequence.

The oligonucleotide primers may be prepared using any suitable method, for example, phosphotriester and phosphodiester methods or automated embodiments thereof, the synthesis of oligonucleotides on a modified solid support, the isolation from a biological source (restriction endonuclease digestion), and the generation by enzymatically directed copying of a DNA or RNA template.

One embodiment of the present invention is a method for simultaneously detecting deletions at a plurality of DNA sequences, comprising the steps of: treating said DNA to form single stranded complementary strands; adding a plurality of paired oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense-strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand; annealing the plurality of primers to their complementary sequences; simultaneously extending said plurality of annealed primers from each primer's 3' terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separation from the complement, serving as templates for the synthesis of an extension product from the other primer of each pair; separating said extension products from said templates to produce single-stranded molecules; amplifying said single-stranded molecules by repeating, at least once, said annealing, extending and separating steps; and identifying said amplified extension product from each different sequence.

One preferred embodiment of the present invention is a method for detecting deletions at a plurality of genomic DNA sequences, wherein said sequences are selected from a group of sequences on the X and Y chromosomes. It is preferable to detect hemizygous genes on the X and Y chromosomes, since this increases the level of sensitivity. When the procedure is used to detect the heterozygous state, it requires quantitative measurement, and thus is much less efficient than detecting the presence or absence of sequences as is done for hemizygous genes. For example, if part of an exon has been deleted the multiplex amplification method of the present invention will detect this by either failing to produce an oligonucleotide sequence or by production of an oligonucleotide sequence of a different size. Furthermore multiple exons can be screened at the same time. Thus, it is easy to detect the presence of a deletion.

However, in looking at heterozygous states, where the chromosomes have one normal gene and one deleted gene, the normal gene will produce a normal product, and thus there is the necessity to measure the quantitative difference in the production of extension products.

A second embodiment of the present invention is to permit simultaneous amplification of multiple, possibly unrelated sequences for the purpose of their simultaneous analysis. Such analysis may simply involve the determination of whether exogenous sequences (virus, bacteria or other parasites) are present within a sample of DNA, or might involve the detection of polymorphisms or mutations within a plurality of sequences. The polymorphisms or mutations can be detected by a variety of methods well known to those skilled in the art. The methods include, but are not limited to, direct DNA sequencing, allele-specific oligonucleotide hybridization, and competitive oligonucleotide priming.

The multiplex genomic DNA amplification method is preferably used to detect X-linked diseases resulting from deletions in the genomic DNA sequence. Genetic diseases can be caused by a variety of mechanisms including mutations and deletions. The procedure described herein was developed for detection of genetic diseases which result from deletions within the genome. Examples of some X-linked diseases which are candidates for the use of multiplex genomic DNA amplification are ornithine transcarbamylase deficiency, hypoxanthine phosphoribosyltransferase deficiency, steroid sulfatase deficiency and X-linked muscular dystrophy. Other disorders on the X chromosome or genes on the Y chromosome can also be easily detected. The procedure is also applicable to the detection of any set of known point mutations within a set of genomic sequences. The procedure is also applicable to the simultaneous detection of any set of exogenous DNA sequences in a given DNA sample. The procedure is also applicable to the simultaneous detection of any set of polymorphic or variable tandemly repetitive sequences within a genome.

The advantages of the multiplex amplification system are that numerous diseases or specific DNA sequence alterations can be detected in the same assay. For example, primers to hypoxanthine phosphoribosyltransferase deficiency, steroid sulfatase deficiency, X-linked muscular dystrophy, ornithine transcarbamylase deficiency and other X-linked diseases can all be run simultaneously on the same sample. Furthermore, the multiplex amplification procedure is useful for very large genes with multiple exons, such as the dystrophin gene. Because of the large size of the dystrophin locus, Mullis type PCR amplification is not able to scan the whole gene in one assay. Thus, it is necessary for multiple site amplification within the gene to detect all possible deletions which could result in disease. Deletions at the DMD locus can encompass any of the approximately 60 plus exons which are distributed over more than 2 million bases of DNA. Virtually all of these exons are separated by large introns and so up to 60 separate reactions could be required for complete analysis of DMD deletions. To simplify this task, the present invention of a multiplex genomic DNA amplification for deletion detection can be employed to perform simultaneous examination of multiple exons. For example, oligonucleotide primers flanking separate DMD gene exons can be synthesized and combined and used for multiplex DNA applications. At present, up to at least 7 different DMD gene sequences have been examined simultaneously. The entire procedure for the multiplex amplification from start-up to photography of the results takes less than 5 hours. The relative locations of the amplified regions do not affect the results and exons have been amplified which have been separated by at least 1000 kb. The PCR amplification technique of Mullis is adequate for one and possibly two pair of primers, but when greater than two pairs of primers are used the procedure will not adequately amplify all the appropriate sequences.

One skilled in the art readily appreciates that as more exon gene sequences become available the applicability of this test will expand to examine for deletions in multiple genes at the same time or examine multiple sites within the same gene at the same time. The latter example is important for genes such as dystrophin which are so large that primers annealed to the ends of the gene will not traverse the whole gene sequence. Thus the necessity of doing multiple analysis to detect deletions in different regions of the gene. In addition, as specific mutations within multiple unrelated genes become known, multiplex DNA amplification can be applied to simultaneously assay for the presence of any of these mutations.

Furthermore, as specific or highly variable DNA sequence polymorphisms become known in various genetic loci, multiplex DNA amplification can be used to simultaneously analyze these polymorphisms to determine the haplotype or to determine the identity or source of DNA (genetic fingerprinting).

The number of analyses which can be run simultaneously is unlimited, however, the upper limit is probably about 20 and is dependent on the size differences required for resolution and/or the number of labels or methods which are available to resolve the extension products. The ability to simultaneously amplify only 9 exons would allow the detection of greater than 90% of all known DMD deletions in a single reaction. The ability to simultaneously amplify even as few as 10 exons allows the rapid and simple diagnosis of DMD deletions using only a few separate reactions. Assuming that there are about 60 exons in the DMD gene and that the exons are widely separated such that primers are needed for every exon, a

maximum of 6 separate assays is needed to detect all deletions in this gene. Under the same assumptions the Mullis PCR method would require 60 separate reactions to detect the deletions in this gene. Thus, as the size of the gene increases and the number of exons which cannot be detected together increases the advantages of this method are greatly enhanced. Furthermore, use of an automatic PCR apparatus (such as that produced by Perkin-Elmer/Cetus) and DNA sequencing machines will facilitate resolution and detection of amplified DNA fragments, will help automate the assay and will lead to the method being applied routinely in clinical laboratories without the need for highly trained research personnel.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. In the examples all percentages are by weight, if for solids and by volume if for liquids, and all temperatures are in degrees Celsius unless otherwise noted.

#### EXAMPLE 1

The following conditions are currently in use to perform simultaneous amplification of a plurality of separate genomic regions within the human DMD gene. These conditions may need to be slightly modified depending on the particular regions to be amplified, the number and length of sequences to be amplified, and the choice of oligonucleotide primers. The time of reaction is highly dependent on the overall sequence length. Thus, as the number of amplified sequences increase and/or the length of amplified sequences increases, the time must be increased. The temperature is dependent on the length, the uniqueness of the primer sequence and the relative percentage of GC bases. The longer the primers, the higher the temperature needed. The more unique the sequence, the lower the temperature needed to amplify. GC rich primers need higher temperatures to prevent cross hybridization and to allow unique amplification. However, as the AT percentage increases, higher temperatures cause these primers to melt. Thus, these primers must be lengthened for the reaction to work.

Template DNA was prepared from the tissue chosen for analysis using a variety of well-established methods known to those skilled in the art. Typically, 100  $\mu$ l reaction volumes were utilized. Approximately 500 ng of DNA was added to a solution comprised of the following: 67 mM Tris-HCL [pH 8.8 at 25°]; 6.7 mM magnesium chloride; 16.6 mM ammonium sulfate; 10 mM  $\beta$ -mercaptoethanol; 6.7  $\mu$ M ethylene diamine tetra-acetic acid (EDTA); and 170  $\mu$ g/mL bovine serum albumin. This solution can be prepared beforehand and appears to be stable for very long periods of storage at -70°. The enzyme, Tag polymerase, was added to achieve a final concentration of 100 units/mL. This reaction mixture was gently mixed. The reaction mixture was overlaid with about 50  $\mu$ L of paraffin oil, and the reaction vessel (preferably a 0.5 ml microcentrifuge tube) was centrifuged at 14,000 x g for 10 sec. Amplification was performed either by manually transferring the reaction vessels between glycerol filled heat blocks at the appropriate temperatures, or automatically transferring the reaction vessels with a Perkin-Elmer/Cetus corporation thermocycler using the 'step-cycle' functions. The reaction was controlled by regulated and repetitive temperature changes of various duration. Initially the reaction was heated to 94° for 7 minutes. Subsequently 25 cycles of the following temperature durations were applied: 94° for 1 minute, then 55° for 45 seconds, then 65° for 3 1/2 minutes. Following completion of the final cycle the reaction was incubated at 65° for an additional 7 minutes. Reactions were then stored at 4° until analysis.

Genomic DNA deletions and/or exogenous DNA sequences were determined by examining the amplification products. For example, the lack of an expected amplification product indicates a deletion. Many methods for this determination are known to those skilled in the art. The preferred method involves electrophoresis of about one-twentieth of the reaction on a 1.4% (weight/vol) agarose gel in the following buffer: 40 mM tris-HCl; 20 mM sodium acetate, 1 mM EDTA (adjusted to pH 7.2 with glacial acetic acid), and 0.5  $\mu$ g/ $\mu$ l. of ethidium bromide. Electrophoresis was performed at 3.7 volts/cm for 100 minutes per 14 cm of agarose gel length. Analysis was completed by examining the electrophoresed reaction products on an ultraviolet radiation transilluminator, and the results were photographed for permanent records.

When the analysis requires determination of DNA sequence polymorphisms or mutations within individual amplification products the agarose gel is transferred to an appropriate DNA binding medium such as nitrocellulose using well-established procedures, for example, Southern blotting. Individual DNA sequences within the amplified DNA fragments can be determined by a variety of techniques including allele-specific oligonucleotide hybridization. Alternatively, reaction products may be further analyzed prior to electrophoresis on agarose gel by competitive oligonucleotide primer amplification, using separate allele-specific primers for each amplified DNA sequence of the multiplex amplification reaction products.

A third method for determining DNA sequence differences within individual amplification products does

not require electrophoresis. In this method, aliquots of the multiplex amplification reaction are sequentially applied to an appropriate DNA binding membran such as nitrocellulose, and then each aliquot is analyzed via hybridization with individual members of sets of allele-specific oligonucleotide (ASO) probes, each separate aliquot being hybridized with one member of a pair of ASO probes specific for one member of the multiply amplified DNA sequences.

## EXAMPLE 2

Figure 1 is a schematic representation of the DMD locus. The relative location of the exons used in the DMD gene amplification examples are illustrated.

For detection of DMD, a variety of probes can be used either in individual PCR reactions or in combinations in multiplex PCR reactions. These probes are shown in Table 1.

Table 1

Summary of DMD gene multiplex amplification primer sets.				
Exon and Size		Primer Sequence	Amplified	Deleted
a.	Exon 8 (182bp)	F-GTCCTTTACACACTTTACCTGTTGAG R-GGCCTCATTCTCATGTTCTAATTAG	360 bp	11.3%
b.	Exon 17 (178bp)	F-GACTTTTCGATGTTGAGATTACTTTCCC R-AAGCTTGAGATGCTCTCACCTTTTCC	416 bp	9.4%
c.	Exon 19 (88bp)	F-TTCTACCACATCCCATTCTTCTCCA R-GATGGCAAAAGTGTTGAGAAAAAGTC	459 bp	10.3%
d.	4.1Kb Hind III (148bp)	F-CTTGATCCATATGCTTTTACCTGCA R-TCCATCACCCCTTCAGAACCTGATCT	268 bp	4.0%
e.	0.5Kb Hind III (176bp)	F-AAACATGGAACATCCTTGTTGGGGAC R-CATTCTATTAGATCTGTGCGCCCTAC	547 bp	8.4%
f.	1.2/3.8Kb Hind III (159bp)	F-TTGAATACATTGGTTAAATCCCAACATG R-CCTGAATAAAGTCTTCCTTACCACAC	506 bp	18.2%
g.	Exon 12 (151bp)	F-GATAGTGGGCTTTACTTACATCCTTC R-GAAAGCACGCAACATAAGATACACCT	337 bp	9.6%
Total:				38%

In Table 1 each exon is designated a, b, c, d, e, f, or g and corresponds to the same letter in Fig. 1. When the exon number is known it is listed. If the exon number is not known, the size of the genomic Hind III fragment containing that exon is listed. Also shown is the size of the exon in base pairs (bp). The PCR primer sequences are shown in 5'-3' orientation. The forward primer (F), hybridizes 5' of the exon, and the reverse primer (R), hybridizes 3' of the exon. The size of the amplified fragment obtained with each primer set is also shown.

The percentage of analyzed DMD patients that are deleted for each indicated exon is shown in column four. This total number is less than the sum of the individual exon deletion frequencies because many deletions encompass multiple exons.

In Table 2 are the exon and flanking intron sequences for Exon 17. The exon is from 227 to 402. The primer sequences used to amplify this sequence are 7 to 33 and 396 to 421.



TABLE 2

	5'	10	20	30	40	50
	TAAATTGACT	TTCGATGTTG	AGATTACTTT	CCCTTGCTAT	TTCAGTGAAC	
5	60	70	80	90	100	
	CAAACTTAAG	TCAGATAAAA	CAATTTTATT	TGGCTTCAAT	ATGGTGCTAT	
	110	120	130	140	150	
	TTTGATCTGA	AGGTCAATCT	ACCAACAAGC	AAGAACAGTT	TCTCATTATT	
	160	170	180	190	200	
10	TTCTTTTGCC	ACTCCAAGCA	GTCTTTACTG	AAGTCTTTCG	AGCAATGTCT	
	210	220	230	240	250	
	GACCTCTGTT	TCAATACTTC	TCACAGATTT	CACAGGCTGT	CACCACCACT	
	260	270	280	290	300	
	CAGCCATCAC	TAACACAGAC	AACTGTAATG	GAAACAGTAA	CTACGGTGAC	
15	310	320	330	340	350	
	CACAAGGGAA	CAGATCCTGG	TAAAGCATGC	TCAAGAGGAA	CTTCCACCAC	
	360	370	380	390	400	
	CACCTCCCCA	AAAGAAGAGG	CAGATTACTG	TGGATTCTGA	AATTAGGAAA	
	410	420	430	440	450	
20	AGGTGAGAGC	ATCTCAAGCT	TTTATCTGCA	AATGAAGTGG	AGAAAACCTCA	
	460	470	480	490	500	
	TTTACAGCAG	TTTTGTTGGT	GGTGTTTTCA	CTTCAGCAAT	ATTTCCAGAA	
25						
	510	520	530	540	550	
	TCCTCGGGTA	CCTGTAATGT	CAGTTAATGT	AGTGAGAAAA	ATTATGAAGT	
	560	570	580	590	600	
30	ACATTTTAAA	ACTTTCACAA	GAAATCACTA	TCGCAACAGA	AACTAAATGC	
	610	620	630	640	650	
	TTAATGGAAA	TGGTGTTTTT	TGGGGTGAAA	GAAGAACTA	TAGAAACTAT	
	660	670	680	690	700	
	AGGTGATAAA	CTACTGTGGT	AGCATTTTAA	TCCTAAAAGT	TTCTTCTTTT	
	710	720	730	740	750	
35	CTTTTTTTTTT	TTTCTTCCTT	ATAAAGGGCC	TGCTTGTTGA	GTCCCTAGTT	
	760	770	780	790	800	
	TTGCATTAAA	TGTCTTTTTT	TTCCAGTAAC	GGAAAGTGCA	TTTTTCATGAA	
	810	820	830	840	850	
40	GAAGTACACC	TATAATAGAT	GGGATCCATC	CTGGTAGTTT	ACGAGAACAT	
	860	870	880	890	900	
	GATGTCTCAG	TCTGCGCATC	CTAAATCAGG	AGTAATTACA	GAACACATTT	
	910	920	930	940	950	
	CCTGTTCTTT	GATATTTATA	AAGTCTTATC	TTGAAGGTGT	TAGAATTTTT	
	960	970	980	990	1000	
45	AACTGATCTT	TTTGTGACTA	TTCAGAATTA	TGCATTTTAG	ATAAGATTAG	
	1010	1020	1030	1040		
	GTATTATGTA	AATCAGTGGA	TATATTAAAT	GATGGCAATA	A-3'	

50 In Table 3 is the exon and flanking intron sequences for Exon d of Table 1 [or, the exon located on a 4.1 kb Hind III fragment]. The exon is from 295 to 442. The primer sequences used to amplify this sequence are 289 to 293 and 512 to 536.

55

TABLE 3

	5'	10	20	30	40	50
	TGTCCAAAT	AGTTGACTTT	CTTTCCTTAA	TCAATAAATA	TATTACTTTA	
5	60	70	80	90	100	
	AAGGGAAAA	TTGCAACCTT	CCATTTAATA	TCAGCTTTAT	ATTGAGTATT	
	110	120	130	140	150	
	TTTTTAAAT	GTTGTGTGTA	CATGCTAGGT	GTGTATATTA	ATTTTTATTT	
	160	170	180	190	200	
10	GTTACTTGAA	ACTAAACTCT	GCAAAATGCAG	GAAACTATCA	GAGTGATATC	
	210	220	230	240	250	
	TTTGTCAGTA	TAACCAAAAA	ATATACGCTA	TATCTCTATA	ATCTGTTTTA	
	260	270	280	290	300	
	CATAATCCAT	CTATTTTCT	TGATCCATAT	GCTTTTACCT	GCAGGCGATT	
15	310	320	330	340	350	
	TGACAGATCT	GTTGAGAAAT	GGCGGCGTTT	TCATTATGAT	ATAAAGATAT	
	360	370	380	390	400	
	TTAATCAGTG	GCTAACAGAA	GCTGAACAGT	TTCTCAGAAA	GACACAAATT	
	410	420	430	440	450	
20	CCTGAGAATT	GGGAACATGC	TAAATACAAA	TGGTATCTTA	AGGTAAGTCT	
	460	470	480	490	500	
	TTGATTTGTT	TTTTCGAAAT	TGTATTTATC	TTCAGCACAT	CTGGACTCTT	
25						
	510	520	530	540	550	
	TAACTTCTTA	AAGATCAGGT	TCTGAAGGGT	GATGGAAATT	ACTTTTGACT	
	560	570	580			
30	GTTGTTGTCA	TCATTATATT	ACTAGAAAGA	AAA-3'		

In Table 4 is the exon and flanking intron sequences for Exon e Table 1 [0.5 Kb Hind III fragment exon]. The exon is from 396 to 571. The primer sequences used to amplify this sequence are 51 to 75 and 572 to 597.

TABLE 4

5'	10	20	30	40	50
ACCCAAATAC	TTTGTTTCATG	TTTAAATTTT	ACAACATTTT	ATAGACTATT	
60	70	80	90	100	
AAACATGGAA	CATCCTTGTTG	GGGACAAGAA	ATCGAATTTG	CTCTTGAAAA	
110	120	130	140	150	
GGTTTCCAAC	TAATTGATTT	GTAGGACATT	ATAACATCCT	CTAGCTGACA	
160	170	180	190	200	
AGCTTACAAA	AATAAAACT	GGAGCTAACC	GAGAGGGTGC	TTTTTCCCT	
210	220	230	240	250	
GACACATAAA	AGGTGTCTTT	CTGTCTTGTA	TCCTTTGGAT	ATGGGCATGT	
260	270	280	290	300	
CAGTTTCATA	GGGAAATTTT	CACATGGAGC	TTTTGTATTT	CTTCTTTTGC	
310	320	330	340	350	
CAGTACAAC	GCATGTGGTA	GCACACTGTT	TAATCTTTTC	TCAAATAAAA	
360	370	380	390	400	
AGACATGGGG	CTTCATTTTT	GTTTTGCCTT	TTTGGTATCT	TACAGGAACT	
410	420	430	440	450	
CCAGGATGGC	ATTGGGCAGC	GGCAAACGT	TGTCAGAACA	TTGAATGCAA	
460	470	480	490	500	
CTGGGGAAGA	AATAATTCAG	CAATCCTCAA	AAACAGATGC	CAGTATTCTA	
510	520	530	540	550	
CAGGAAAAAT	TGGGAAGCCT	GAATCTGCGG	TGGCAGGAGG	TCTGCAAACA	
560	570	580	590	600	
GCTGTCAGAC	AGAAAAAAGA	GGTAGGGCGA	CAGATCTAAT	AGGAATGAAA	
610	620				
ACATTTTAGC	AGACTTTTTA	AGCTT-3'			

30 In Table 5 is the exon and flanking intron sequences for Exon f, Table 1 [overlaps the 1.2 Kb and 3.8 Kb Hind III fragments]. The exon is from 221 to 406. The primer sequences used to amplify this sequence are 26 to 53 and 516 to 541.

TABLE 5

5'	10	20	30	40	50
TTTTGTAGAC	GGTTAATGAA	TAATTTTGAA	TACATTGGTT	AAATCCCAAC	
60	70	80	90	100	
ATGTAATATA	TGTAAATAAT	CAATATTATG	CTGCTAAAAT	AACACAAATC	

	110	120	130	140	150
	AGTAAGATTC	TGTAATATTT	CATGATAAAT	AACTTTTGAA	AATATATTTT
	160	170	180	190	200
5	TAAACATTTT	GCTTATGCCT	TGAGAATTAT	TTACCTTTTT	AAAATGTATT
	210	220	230	240	250
	TTCCTTTCAG	GTTTCCAGAG	CTTTACCTGA	GAAACAAGGA	GAAATTGAAG
	260	270	280	290	300
	CTCAAATAAA	AGACCTTGGG	CAGCTTGAAA	AAAAGCTTGA	AGACCTTGAA
10	310	320	330	340	350
	GAGCAGTTAA	ATCATCTGCT	GCTGTGGTTA	TCTCCTATTA	GGAATCAGTT
	360	370	380	390	400
	GGAAATTTAT	AACCAACCAA	ACCAAGAAGG	ACCATTTGAC	GTTAAGGTAG
	410	420	430	440	450
15	GGGAACTTTT	TGCTTTAATA	TTTTTGCTCT	TTTTAAGAAA	AATGGCAATA
	460	470	480	490	500
	TCACTGAATT	TTCTCATTG	GTATCATTAT	TAAAGACAAA	ATATTACTTG
	510	520	530	540	550
	TTAAAGTGTG	GTAAGGAAGA	CTTTATTTCAG	GATAACCACA	ATAGGCACAG
20	560	570	580	590	600
	GGACCACTGC	AATGGAGTAT	TACAGGAGGT	TGGATAGAGA	GAGATTGGGC
	610	620	630	640	650
	TCAACTCTAA	ATACAGCACA	GTGGAAGTAG	GAATTTATAG	C-3'

25 In Table 6 is the exon and flanking intron sequences for Exon 12. The exon is from 180 to 329. The primer sequences used to amplify this sequence are 27 to 52 and 332 to 357.

TABLE 6

30	5'	10	20	30	40	50
	TGAGAAATAA	TAGTTCGGG	GTGACTGATA	GTGGGCTTTA	CTTACATCCT	
	60	70	80	90	100	
35	TCTCAATGTC	CAATAGATGC	CCCCAAATGC	GAACATTCCA	TATATTATAA	
	110	120	130	140	150	
	ATTCTATTGT	TTTACATTGT	GATGTTTCAGT	AATAAGTTGC	TTTCAAAGAG	
	160	170	180	190	200	
	GTCATAATAG	GCTTCTTTCA	AATTTTCAGT	TTACATAGAG	TTTTAATGGA	
40	210	220	230	240	250	
	TCTCCAGAAT	CAGAAACTGA	AAGAGTTGAA	TGACTGGCTA	ACAAAACAGA	
	260	270	280	290	300	
	AGAAAGAACA	AGGAAAATGG	AGGAAGAGCC	TCTTGGACCT	GATCTTGAAG	
	310	320	330	340	350	
45	ACCTAAAACG	CCAAGTACAA	CAACATAAGG	TAGGTGTATC	TTATGTTGCG	
	360	370	380	390	400	
	TGCTTTCTAC	TAGAAAGCAA	ACTCTGTGTA	TAGTACCTAT	ACACAGTAAC	
	410	420	430	440	450	
	ACAGATGACA	TGGTTGATGG	GAGAGAATTA	AAACTTAAAG	TCAGCCATAT	
	460	470	480	490	500	
50	TTTAAAAATT	ATTTTACCT	AATTGTTTTT	GCAATCTTTG	TTGCCAATGG	
	510	520	530	540	550	
	CCTTGAATAA	GTCCCCTCCA	AAATTCAGGT	GATTGTATTA	GGAGATGGAA	

55

	560	570	580	590	600
	TATTTAAGGG	TGAATAATCC	ATCAGGGCTC	CTCCCTTAAG	AATAGGATCA
	610	620	630	640	650
5	AGTCCCATAT	AAAAGAGGCT	TCACACAGTG	TTCTCCTATC	TCTTGACCCT
	660	670	680	690	700
	CCACCATGCA	CCACCATGTG	AAAACCTCTGT	GAAAAGGCC	TCACCAGATG
	710	720	730	740	750
	CTAACATCTT	GATCTTGGAT	TTCCCAAAC	CGAGAACTGT	GAAAAAATAA
	760	770	780	790	800
10	AGGTACATTC	TTCTTAAATT	ACCTCATTCT	CATTTAAACA	CACAAAGTGC
	810				
	ACACATAGCT	G-3'			

15 In Table 7 is the exon and flanking intron sequences for the Exon located on a 10 Kb Hind III fragment. The exon is from 1 to 150.

TABLE 7

20	5'	10	20	30	40	50
	TTACTGGTGG	AAGAGTTGCC	CCTGCGCCAG	GGAATTCTCA	AACAATTAA	
	60	70	80	90	100	
	TGAAACTGGA	GGACCCGTGC	TTGTAAGTGC	TCCATAAGC	CCAGAAGAGC	
25	110	120	130	140	150	
	AAGATAAACT	TGAAAATAAG	CTCAAGCAGA	CAAATCTCCA	GTGGATAAAG	
	160	170	180	190	200	
	GTTAGACATT	AACCATCTCT	TCCGTCACAT	GTGTTAAATG	TTGCAAGTAT	
	210	220	230	240	250	
30	TTGTATGTAT	TTTGTTCCT	GGGTGCTTCA	TTGGTCGGGG	AGGAGGCTGG	
	260	270	280			
	TATGTGGATT	GTTGTTTTGT	TTTGTTTTTT-3'			

35 In Table 8 is the exon and flanking intron sequences for the Exon located on a 1.6 Kb Hind III fragment from 512 to 622.

TABLE 8

40	5'	10	20	30	40	50
	AAGCTTTGAT	ACTGTGCTTT	AAGTGTTTAC	CCTTTGGAAA	GAAAAATAATT	
	60	70	80	90	100	
	TTGACAGTGA	TGTAGAAATA	ATTATTTGAT	ATTTATTTCA	AAACAAAATT	
45	110	120	130	140	150	
	TATATCCAAT	ACTAAACACA	GAATTTTGTA	AAACAATAAG	TGTATAAAGT	
	160	170	180	190	200	
	AAAATGAACA	TTAGGATTAT	TGAGATTATT	GTAGCTAAAA	CTAGTGTTTA	
	210	220	230	240	250	
50	TTCATATAAA	TTATGTTAAT	AAATTGTATT	GTCATTATTG	CATTTTACTT	
	260	270	280	290	300	
	TTTTGAAAAG	TAGTTAATGC	CTGTGTTTCT	ATATGAGTAT	TATATAATTC	

55

	310	320	330	340	350
	AAGAAGATAT	TGGATGAATT	TTTTTTTTTAA	GTTTAATGTG	TTTCACATCT
	360	370	380	390	400
5	CTGTTTCTTT	TCTCTGCACC	AAAAGTCACA	TTTTTGTGCC	CTTATGTACC
	410	420	430	440	450
	AGGCAGAAAT	TGATCTGCAA	TACATGTGGA	GTCTCCAAGG	GTATATTTAA
	460	470	480	490	500
	ATTTAGTAAT	TTTATTGCTA	ACTGTGAAGT	TAATCTGCAC	TATATGGGTT
10	510	520	530	540	550
	CTTTTCCCCA	GGAAACTGAA	ATAGCAGTTC	AAGCTAAACA	ACCGGATGTG
	560	570	580	590	600
	GAAGAGATTT	TGTCTAAAGG	GCAGCATTTG	TACAAGGAAA	AACCAGCCAC
	610	620	630	640	650
15	TCAGCCAGTG	AAGGTAATGA	AGCAACCTCT	AGCAATATCC	ATTACCTCAT
	660	670	680	690	700
	AATGGGTTAT	GCTTCGCCTG	TTGTACATTT	GCCATTGACG	TGGACTATTT
	710	720	730	740	750
	ATAATCAGTG	AAATAACTTG	TAAGGAAATA	CTGGCCATAC	TGTAATAGCA
20	760	770	780	790	800
	GAGGCAAAGC	TGTCTTTTGT	ATCAGCATAT	CCTATTTATA	TATTGTGATC
	810	820	830	840	
	TTAAGGCTAT	TAACGAGTCA	TTGCTTTAAA	GGACTCATTT	CTGTC-3'

25

In Table 9 is the exon and flanking intron sequences for the Exon located on a 3.1 Kb Hind III fragment. The exon is from 519 to 751.

TABLE 9						
30	5'	103	113	123	133	143
	CCCATCTTGT	TTTGCCTTTG	TTTTTTCTTG	AATAAAAAAA	AAATAAGTAA	
	153	163	173	183	193	
	AATTTATTTT	CCTGGCAAGG	TCTGAAAAC	TTTGTTTTCT	TTACCACTTC	
35	203	213	223	233	243	
	CACAATGTAT	ATGATTGTTA	CTGAGAAGGC	TTATTTAACT	TAAGTTACTT	
	253	263	273	283	293	
	GTCCAGGCAT	GAGAATGAGC	AAAATCGTTT	TTTAAAAAAT	TGTTAAATGT	
	303	313	323	333	343	
40	ATATTAATGA	AAAGGTTGAA	TCTTTTCATT	TTCTACCATG	TATTGCTAAA	
	353	363	373	383	393	
	CAAAGTATCC	ACATTGTTAG	AAAAAGATAT	ATAATGTCAT	GAATAAGAGT	
	403	413	423	433	443	
	TTGGCTCAAA	TTGTTACTCT	TCAATTAAAT	TTGACTTATT	GTTATTGAAA	
45	453	463	473	483	493	
	TTGGCTCTTT	AGCTTGTGTT	TCTAATTTTT	CTTTTTCTTC	TTTTTTCCTT	
	503	513	523	533	543	
	TTTGCAAAAA	CCCAAAATAT	TTTAGCTCCT	ACTCAGACTG	TTACTCTGGT	
	553	563	573	583	593	
50	GACACAACCT	GTGGTTACTA	AGGAAACTGC	CATCTCCAAA	CTAGAAATGC	
	603	613	623	633	643	
	CATCTTCCTT	GATGTTGGAG	GTACCTGCTC	TGGCAGATTT	CAACCGGGCT	
	653	663	673	683	693	
	TGGACAGAAC	TTACCGACTG	GCTTTCTCTG	CTTGATCAAG	TTATAAAATC	

55

	703	713	723	733	743
	ACAGAGGGTG	ATGGTGGGTG	ACCTTGAGGA	TATCAACGAG	ATGATCATCA
	753	763	773	783	793
6	AGCAGAAGGT	ATGAGAAAAA	ATGATAAAAG	TTGGCAGAAG	TTTTTCTTTA
	803	813	823	833	843
	AAATGAAGAT	TTTCCACCAA	TCACTTTACT	CTCCTAGACC	ATTTCCCACC
	853	863	873	883	893
	AGTTCTTAGG	CAACTGTTTC	TCTCTCAGCA	AACACATTAC	TCTCACTATT
10	903	913	923	933	943
	CAGCCTAAGT	ATAATCAGGT	ATAAATTAAT	GCAAATAACA	AAAGTAGCCA
	953	963	973	983	993
	TACATTAAAA	AGGAAAATAT	ACAAAAAATA	AAAAAATAAA	AAGCCAGAAA
	1003	1013			
15	CCTACAGAAT	AGTGCTCTAG	TAATTAC-3'		

In Table 10 is the exon and flanking intron sequences for the Exon located on a 1.5 Kb Hind III fragment. The exon is from 190 to 337.

20

TABLE 10

	5'	10	20	30	40	50
25	ATCTCTATCA	TTAGAGATCT	GAATATGAAA	TACTTGTCAA	AGTGAATGAA	
	60	70	80	90	100	
	AATTTNNTAA	ATTATGTATG	GTTAACATCT	TTAAATTGCT	TATTTTATAA	
	110	120	130	140	150	
	TTGCCATGTT	TGTGTCCCAG	TTTGCATTAA	CAAATAGTTT	GAGAACTATG	
	160	170	180	190	200	
30	TTGGAAAAAA	AAATAACAAT	TTTATTCTTC	TTTCTCCAGG	CTAGAAGAAC	
	210	220	230	240	250	
	AAAAGAATAT	CTTGTCAGAA	TTTCAAAGAG	ATTTAAATGA	ATTTGTTTAA	
	260	270	280	290	300	
35	TGGTTGGAGG	AAGCAGATAA	CATTGCTAGT	ATCCCACTTG	AACCTGGAAA	
	310	320	330	340	350	
	AGAGCAGCAA	CTAAAAGAAA	AGCTTGAGCA	AGTCAAGGTA	ATTTTATTTT	
	360	370	380	390	400	
	CTCAAATCCC	CCAGGGCCTG	CTTGCAATAA	GAAGTATATG	AATCTATTTT	
	410	420	430	440	450	
40	TTAATTC AAT	CATTGGTTTT	CTGCCCATT A	GGTTATTCAT	AGTTCCTTGC	
	460	470	480	490	500	
	TAAAGTGTTT	TTCTCAC AAC	TTTATTTCTT	CTTAACCCTG	CAGTTCTGAA	
	510	520	530	540	550	
	CCAGTG CACA	TAAGAACATA	TGTATATATG	TGTGTGTGTG	TATTTATATA	
45	560	570	580	590	600	
	TACACACACA	CATATTGCAT	CTATACATCT	ACACATATAG	ATGTATAGAT	
	610	620	630	640	650	
	TCAATATGTC	TAAAAATGTA	TATAATTCAC	AGTTTTTATC	TTTGATTGTA	
	660	670	680			
50	ATATTTAAGG	GACTGAGACT	CACACTCATA	TACTTTT-3'		

55

EXAMPLE 3

Prenatal Diagnosis and Detection of DMD Using PCR

5 An example of prenatal diagnosis with PCR deletion detection is demonstrated using synthesized oligonucleotide primers (set b, Table 1). This primer set corresponds to the intron sequences flanking exon 17 of the human DMD gene, a region which has been isolated and sequenced (Table 2).

The results of this analysis are shown in figure 3. The PCR products (one-twentieth of the total reaction) were obtained with template DNA isolated from a control male □, the male fetus being diagnosed Δ, the DMD carrier mother (O) and an affected male brother of the fetus ■. Also shown is a DNA molecular weight standard (MW; Hae III digested φX174 DNA). The results demonstrate that the affected male carries a deletion of exon 17, which was not amplified, but that the fetus does not carry the deletion and is therefore unaffected. These results indicate that PCR is useful in the diagnosis of DMD cases containing a deletion involving this exon.

EXAMPLE 4Multiplex Detection

An example of multiplex detection is shown in Figures 3A and 3B.

25 This analysis was done using six primer pairs (sets a-f, Table 1) and the conditions described in Example 1. Automatic rather than manual amplification was performed. These oligonucleotide primers represent the flanking regions of six separate DMD gene exons. They were combined into a reaction vial and used for multiplex genomic DNA amplifications. Template DNA was isolated from lymphoblasts (from blood sample). Analysis was by agarose gel electrophoresis.

30 When non-deleted DNA was used as a template, the six dispersed regions of the DMD gene were simultaneously and specifically amplified (Figure 3A, Sample #534). Discrete deletions, which were detected with this method, are shown in Figures 3A and 3B. Several DNA samples containing normal, partial or total DMD gene deletions are shown. Figures 3A and 3B also show a DNA molecular weight standard (MW: Hae III digested φX174 DNA), and a negative control (-) where no template DNA was added to the reactions. Figure 3A also indicates which amplified DNA fragment corresponds to which exon (a-f) of Figure 1.

EXAMPLE 5Prenatal Diagnosis

45 Multiplex PCR has been used successfully in several prenatal diagnoses. The conditions are as described above in Example 1. Figure 4 shows Multiplex DNA amplification for prenatal diagnosis of DMD. Shown are the results of amplification using DNA from affected males (AM; lymphoblast DNA) and male fetuses (MF; cultured amniotic fluid cell DNA) from six different families. Analysis was as described in Example 1. Both the affected male and the fetal DNA of DRL #s 521 and 531 display a deletion of region f (Figure 1). Thus these fetuses were diagnosed as affected. In DRL # 43C the affected male is deleted for all regions except f, while the fetus is unaffected. The affected male in DRL #483 is deleted for region a, while the male fetus is unaffected. Neither of the samples from DRL #s 485 or 469 displayed a deletion with this technique. Thus, if a deletion defect causes DMD in these families it occurred in an untested exon.

EXAMPLE 6



Prenatal diagnosis using multiplex DNA amplification of chorionic villus specimen (CVS) DNA

Figure 5 demonstrates Multiplex DNA amplification from CVS DNA. Both the affected male (AM; lymphoblast DNA) and the male fetus (MF; CVS DNA) from DRL # 92 display a deletion of regions e and f (Fig. 1). Thus the fetus was diagnosed as affected. CVS DNA from DRL # 120 did not display a deletion with this technique. Samples were analyzed as described in Example 1. These results demonstrate that the multiplex amplification technique works well for prenatal diagnosis when CVS DNA is used as the template for amplification.

EXAMPLE 7Multiplex amplification of seven separate exons of the DMD gene

This example demonstrates that seven separate DNA sequences can be simultaneously amplified using the multiplex amplification technique. Conditions were as described in Example 1. Primer sets a-g (Table 1) were added to the reaction. Thus seven exon regions of the DMD gene (Figure 1) were amplified (Figure 6).

EXAMPLE 8Multiplex DNA amplification for the simultaneous detection of mutations leading to multiple common genetic diseases

This example describes how the multiplex amplification technique can be used to simultaneously screen a newborn male for any of the most common mutations leading to DMD, sickle-cell anemia and  $\alpha_1$ -antitrypsin deficiency. In this assay any or all of the primers sets listed in Table 1 can be used for multiplex DNA amplification to diagnose the majority of possible DMD gene deletions. Additionally, primer sets can be added to the amplification reaction to identify mutations leading to additional genetic diseases. Other primer sets include:

A.

5'-TGGTCTCCTTAAACCTGTCTT-3'

5'-ACACAACTGTGTTCCTAG-3'

These oligonucleotides amplify a 167 bp segment of the human  $\beta$ -globin gene, containing the DNA base that is mutated in  $\beta^s$  (sickle-cell) hemoglobinopathy. The presence or absence of the mutant  $\beta^s$  sequence is then determined either by separate dot blot or Southern blot hybridization of the multiplex amplification reaction with each of two labelled allele-specific oligonucleotide (ASO) probes specific for the normal or  $\beta^s$  sequence. The sequence of these two ASO probes is:

1) Normal: 5'-CTCCTGAGGAGA-3'

2)  $\beta^s$ : 5'-CTCCTGTGGAGA-3'

If dot blot hybridization is used, a separate application of DNA from the multiplex amplification reaction to a DNA membrane, such as nitrocellulose, is required for each probe that will be used in the hybridization. Hybridization of each labelled probe, whether the probes are complementary to individual alleles of a given gene or to separate genes, must be performed individually. Alternatively and preferably, two aliquots of the amplification reaction are separately electrophoresed on agarose gels and transferred to nitrocellulose or a similar membrane using Southern analysis. Each of the two Southern blots are then hybridized with one member of each labelled set of specific ASO primers. Thus each known mutant or normal allele of each DNA fragment amplified in the multiplex reaction can be determined.

In addition to the above described primer sets the following oligonucleotide primers can also be added to the amplification procedure:

B.

5'-ACGTGGAGTGACGATGCTCTTCCC-3'  
5'-GTGGGATTCACCACTTTTCCC-3'

These primers produce a 450 bp DNA fragment containing the DNA base change that produces the Z allele of the  $\alpha_1$ -antitrypsin gene and leads to  $\alpha_1$ -antitrypsin deficiency. The Z allele and the normal M allele are distinguished from other alleles in the multiplex amplification reaction by hybridization with the ASO probes:

- 1) Normal (M) allele: 5'-ATCGACGAGAAA-3'
- 2) Mutant (Z) allele: 5'-ATCGACAAGAAA-3'

Hybridization analysis is performed in parallel with the  $\beta$ -globin probes as described above.

In addition, the oligonucleotides

C.  
5'-GAAGTCAAGGACACCGAGGAA-3'  
5'-AGCCCTCTGGCCAGTCCTAGTG-3'

can also be added to the multiplex reaction to produce a 340 bp DNA region of the  $\alpha_1$ -antitrypsin gene that contains the DNA base change that produces the S allele and leads to  $\alpha_1$ -antitrypsin deficiency. The S allele is distinguished from other alleles in the multiplex amplification as described above for the  $\beta^s$  and Z alleles by using the two ASO probes specific for the M and S allele:

Normal (M) allele 5'-ACCTGGAAAATG-3'  
Mutant (S) allele 5'-ACCTGGTAAATG-3'

Using the primers described in Table 1 and in A, B and C of this example the common mutations leading to DMD, sickle cell anemia and  $\alpha_1$ -antitrypsin deficiency can be simultaneously determined.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well, those inherent therein. The methods procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary, and are not intended as limitations on the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

### Claims

1. A method for simultaneously detecting deletions at a plurality of DNA sequences, comprising the steps of:  
treating said DNA to form single-stranded complementary strands;  
adding a plurality of paired oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense-strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand;  
annealing the plurality of primers to their complementary sequences;  
simultaneously extending said plurality of annealed primers from each primer's 3' terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separation from their complement, serving as templates for the synthesis of an extension product from the other primer of each pair;  
separating said extension products from said templates to produce single-stranded molecules;  
amplifying said single stranded molecules by repeating, at least once, said annealing, extending and separating steps; and  
identifying said amplified extension products from each different sequence.
2. The method of Claim 1 for detecting deletions at a plurality of genomic DNA sequences, wherein said sequences are selected from the group of sequences on the X and Y chromosomes.
3. The method of Claim 2 for the detection of X-linked disease, wherein said genomic DNA sequences contain a deletion that causes a genetic disease.
4. The method of Claim 3 for the detection of said X-linked genetic diseases selected from the group consisting of ornithine transcarbamylase deficiency, hypoxanthine phosphoribosyltransferase deficiency, steroid sulfatase deficiency and X-linked muscular dystrophy.
5. The method of Claim 4 for the detection of X-linked muscular dystrophy, wherein said plurality of paired primers are complementary to different sequences within the gene coding for the dystrophin protein.
6. The method of Claim 5, wherein the plurality of paired primers is selected from the group consisting of:

- (1) 5'-GACTTTGATGTTGAGATTACTTTCCC-3'  
 (2) 5'-AAGCTTGAGATGCTCTCACCTTTTCC-3',  
 (1) 5'-GTCCTTTACACACTTTACCTGTTGAG-3'  
 (2) 5'-GGCCTCATTCTCATGTTCTAATTAG-3',  
 5 (1) 5'-AAACATGGAACATCCTTGTGGGGAC-3',  
 (2) 5'-CATTCTATTAGATCTGTGCGCCTAC-3',  
 (1) 5'-GATAGTGGGCTTTACTTACATCCTTC-3',  
 (2) 5'-GAAAGCACGCAACATAAGATACACCT-3',  
 (1) 5'-CTTGATCCATATGCTTTTACCTGCA-3',  
 10 (2) 5'-TCCATCACCTTCAGAACCTGATCT-3',  
 (1) 5'-GAATACATTGGTTAAATCCCAACATG-3',  
 (2) 5'-CCTGAATAAAGTCTTCCTTACCACAC-3', and  
 (1) 5'-TTCTACCACATCCCATTCTTCCA-3'  
 (2) 5'-GATGGCAAAAGTGTGAGAAAAAGTC-3'.

15 7. The method of Claim 3, wherein said genomic DNA is from fetal tissue.

8. The method of Claim 1 for detecting deletions at a plurality of genomic DNA sequences, wherein the plurality of paired primers is selected from the group consisting of:

- (1) 5'-GACTTTGATGTTGAGATTACTTTCCC-3'  
 (2) 5'-AAGCTTGAGATGCTCTCACCTTTTCC-3',  
 20 (1) 5'-GTCCTTTACACACTTTACCTGTTGAG-3'  
 (2) 5'-GGCCTCATTCTCATGTTCTAATTAG-3',  
 (1) 5'-AAACATGGAACATCCTTGTGGGGAC-3',  
 (2) 5'-CATTCTATTAGATCTGTGCGCCTAC-3',  
 (1) 5'-GATAGTGGGCTTTACTTACATCCTTC-3',  
 25 (2) 5'-GAAAGCACGCAACATAAGATACACCT-3',  
 (1) 5'-CTTGATCCATATGCTTTTACCTGCA-3',  
 (2) 5'-TCCATCACCTTCAGAACCTGATCT-3',  
 (1) 5'-GAATACATTGGTTAAATCCCAACATG-3',  
 (2) 5'-CCTGAATAAAGTCTTCCTTACCACAC-3',  
 30 (1) 5'-TTCTACCACATCCCATTCTTCCA-3'  
 (2) 5'-GATGGCAAAAGTGTGAGAAAAAGTC-3',  
 (1) 5'-TGGTCTCCTTAAACCTGTCTT-3',  
 (2) 5'-ACACAACCTGTGTTCACTAG-3',  
 (1) 5'-ACGTGGAGTGACGATGCTCTTCCC-3',  
 35 (2) 5'-GTGGGATTCACCACTTTTCCC-3', and  
 (1) 5'-GAAGTCAAGGACACCGAGGAA-3',  
 (2) 5'-AGCCCTCTGGCCAGTCCTAGTG-3'.

9. A DNA sequence of the formula:

40	5'	10	20	30	40	50
	TAAATTGACT	TTCGATGTTG	AGATTACTTT	CCCTTGCTAT	TTCAGTGAAC	
	60	70	80	90	100	
	CAAACTTAAG	TCAGATAAAA	CAATTTTATT	TGGCTTCAAT	ATGGTGCTAT	
45	110	120	130	140	150	
	TTTGATCTGA	AGGTCAATCT	ACCAACAAGC	AAGAACAGTT	TCTCATTATT	
	160	170	180	190	200	
	TTCTTTTGCC	ACTCCAAGCA	GTCTTTACTG	AAGTCTTTCG	AGCAATGTCT	
	210	220	230	240	250	
50	GACCTCTGTT	TCAATACTTC	TCACAGATTT	CACAGGCTGT	CACCACCACT	
	260	270	280	290	300	
	CAGCCATCAC	TAACACAGAC	AACTGTAATG	GAAACAGTAA	CTACGGTGAC	

55

	310	320	330	340	350
	CACAAGGGAA	CAGATCCTGG	TAAAGCATGC	TCAAGAGGAA	CTTCCACCAC
	360	370	380	390	400
5	CACCTCCCCA	AAAGAAGAGG	CAGATTACTG	TGGATTCTGA	AATTAGGAAA
	410	420	430	440	450
	AGGTGAGAGC	ATCTCAAGCT	TTTATCTGCA	AATGAAGTGG	AGAAAACCTCA
	460	470	480	490	500
	TTTACAGCAG	TTTTGTTGGT	GGTGTTTTCA	CTTCAGCAAT	ATTTCCAGAA
10	510	520	530	540	550
	TCCTCGGGTA	CCTGTAATGT	CAGTTAATGT	AGTGAGAAAA	ATTATGAAGT
	560	570	580	590	600
	ACATTTTAAA	ACTTTCACAA	GAAATCACTA	TCGCAACAGA	AACTAAATGC
	610	620	630	640	650
15	TTAATGGAAA	TGGTGTTTTC	TGGGGTGAAA	GAAGAAACTA	TAGAAACTAT
	660	670	680	690	700
	AGGTGATAAA	CTACTGTGGT	AGCATTTTAA	TCCTAAAAGT	TTCTTTCTTT
	710	720	730	740	750
	CTTTTTTTTT	TTTCTTCCTT	ATAAAGGGCC	TGCTTGTTGA	GTCCCTAGTT
20	760	770	780	790	800
	TTGCATTAAA	TGTCTTTTTT	TTCCAGTAAC	GGAAAGTGCA	TTTTCATGAA
	810	820	830	840	850
	GAAGTACACC	TATAATAGAT	GGGATCCATC	CTGGTAGTTT	ACGAGAACAT
	860	870	880	890	900
25	GATGTCTCAG	TCTGCGCATC	CTAAATCAGG	AGTAATTACA	GAACACATTT
	910	920	930	940	950
	CCTGTCTCTT	GATATTTATA	AAGTCTTATC	TTGAAGGTGT	TAGAATTTTT
	960	970	980	990	1000
	AACTGATCTT	TTTGTGACTA	TTCAGAATTA	TGCATTTTAG	ATAAGATTAG
30	1010	1020	1030	1040	
	GTATTATGTA	AATCAGTGGA	TATATTAAAT	GATGGCAATA	A-3'

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

10. A DNA sequence of the formula:

	5'	10	20	30	40	50
40	TGTCCAAAT	AGTTGACTTT	CTTCTTTTAA	TCAATAAATA	TATTACTTTA	
	60	70	80	90	100	
	AAGGGAAAAA	TTGCAACCTT	CCATTTAAAA	TCAGCTTTAT	ATTGAGTATT	
	110	120	130	140	150	
45	TTTTTAAAT	GTTGTGTGTA	CATGCTAGGT	GTGTATATTA	ATTTTTATTT	
	160	170	180	190	200	
	GTTACTTGAA	ACTAAACTCT	GCAAATGCAG	GAAACTATCA	GAGTGATATC	
	210	220	230	240	250	
	TTTGTCAGTA	TAACCAAAAA	ATATACGCTA	TATCTCTATA	ATCTGTTTTA	
50	260	270	280	290	300	
	CATAATCCAT	CTATTTTCT	TGATCCATAT	GCTTTTACCT	GCAGGCGATT	

	310	320	330	340	350
	TGACAGATCT	GTTGAGAAAT	GGCGGCGTTT	TCATTATGAT	ATAAAGATAT
	360	370	380	390	400
5	TTAATCAGTG	GCTAACAGAA	GCTGAACAGT	TTCTCAGAAA	GACACAAATT
	410	420	430	440	450
	CCTGAGAATT	GGGAACATGC	TAAATACAAA	TGGTATCTTA	AGGTAAGTCT
	460	470	480	490	500
	TTGATTTGTT	TTTTTCGAAAT	TGTATTTATC	TTCAGCACAT	CTGGACTCTT
	510	520	530	540	550
10	TAACTTCTTA	AAGATCAGGT	TCTGAAGGGT	GATGGAAATT	ACTTTTGACT
	560	570	580		
	GTTGTTGTCA	TCATTATATT	ACTAGAAAAGA	AAA-3'	

15 and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

11. A DNA sequence of the formula:

20	5'	10	20	30	40	50
	ACCCAAATAC	TTTGTTCATG	TTTAAATTTT	ACAACATTTT	ATAGACTATT	
	60	70	80	90	100	
	AAACATGGAA	CATCCTTGTG	GGGACAAGAA	ATCGAATTTG	CTCTTGAAAA	
25	110	120	130	140	150	
	GGTTTCCAAC	TAATTGATTT	GTAGGACATT	ATAACATCCT	CTAGCTGACA	
	160	170	180	190	200	
	AGCTTACAAA	AATAAAAAC	GGAGCTAACC	GAGAGGGTGC	TTTTTTCCCT	
	210	220	230	240	250	
30	GACACATAAA	AGGTGTCTTT	CTGTCTTGTA	TCCTTTGGAT	ATGGGCATGT	
	260	270	280	290	300	
	CAGTTTCATA	GGGAAATTTT	CACATGGAGC	TTTTGTATTT	CTTTCTTTGC	
	310	320	330	340	350	
	CAGTACAAC	GCATGTGGTA	GCACACTGTT	TAATCTTTTC	TCAAATAAAA	
35	360	370	380	390	400	
	AGACATGGGG	CTTCATTTTT	GTTTTGCCTT	TTTGGTATCT	TACAGGAAC	
	410	420	430	440	450	
	CCAGGATGGC	ATTGGGCAGC	GGCAAAC	TGTGAGAAC	TTGAATGCAA	
	460	470	480	490	500	
40	CTGGGGAAGA	AATAATTCAG	CAATCCTCAA	AAACAGATGC	CAGTATTCTA	
	510	520	530	540	550	
	CAGGAAAAAT	TGGGAAGCCT	GAATCTGCGG	TGGCAGGAGG	TCTGCAAACA	
	560	570	580	590	600	
	GCTGTCAGAC	AGAAAAAGA	GGTAGGGCGA	CAGATCTAAT	AGGAATGAAA	
45	610	620				
	ACATTTTAGC	AGACTTTTAA	AGCTT-3'			

50 and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

12. A DNA sequence of the formula:

	5'	10	20	30	40	50
		TTTTGTAGAC	GGTTAATGAA	TAATTTTGAA	TACATTGGTT	AAATCCCAAC
		60	70	80	90	100
5		ATGTAATATA	TGTAAATAAT	CAATATTATG	CTGCTAAAAT	AACACAAATC
		110	120	130	140	150
		AGTAAGATTC	TGTAATATTT	CATGATAAAT	AACTTTTGAA	AATATATTTT
		160	170	180	190	200
		TAAACATTTT	GCTTATGCCT	TGAGAATTAT	TTACCTTTTT	AAAATGTATT
		210	220	230	240	250
10		TTCCTTTCAG	GTTTCCAGAG	CTTTACCTGA	GAAACAAGGA	GAAATTGAAG
		260	270	280	290	300
		CTCAAATAAA	AGACCTTGGG	CAGCTTGAAA	AAAAGCTTGA	AGACCTTGAA
		310	320	330	340	350
		GAGCAGTTAA	ATCATCTGCT	GCTGTGGTTA	TCTCCTATTA	GGAATCAGTT
15		360	370	380	390	400
		GGAAATTTAT	AACCAACCAA	ACCAAGAAGG	ACCATTTGAC	GTTAAGGTAG
		410	420	430	440	450
		GGGAACTTTT	TGCTTTAATA	TTTTTGTCTT	TTTTAAGAAA	AATGGCAATA
		460	470	480	490	500
20		TCACTGAATT	TTCTCATTTG	GTATCATTAT	TAAAGACAAA	ATATTACTTG
		510	520	530	540	550
		TTAAAGTGTG	GTAAGGAAGA	CTTTATTCAG	GATAACCACA	ATAGGCACAG
		560	570	580	590	600
25		GGACCACTGC	AATGGAGTAT	TACAGGAGGT	TGGATAGAGA	GAGATTGGGC
		610	620	630	640	650
		TCAACTCTAA	ATACAGCACA	GTGGAAGTAG	GAATTTATAG	C-3'

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

13. A DNA sequence of the formula:

	5'	10	20	30	40	50
35		TGAGAAATAA	TAGTTCCGGG	GTGACTGATA	GTGGGCTTTA	CTTACATCCT
		60	70	80	90	100
		TCTCAATGTC	CAATAGATGC	CCCCAAATGC	GAACATTCCA	TATATTATAA
		110	120	130	140	150
40		ATTCTATTGT	TTTACATTGT	GATGTTTCA	AATAAGTTGC	TTTCAAAGAG
		160	170	180	190	200
		GTCATAATAG	GCTTCTTTCA	AATTTTCAGT	TTACATAGAG	TTTAAATGGA
		210	220	230	240	250
		TCTCCAGAAT	CAGAACTGA	AAGAGTTGAA	TGACTGGCTA	ACAAAACAGA
45						
50						
55						

	260	270	280	290	300
	AGAAAGAACA	AGGAAAATGG	AGGAAGAGCC	TCTTGGACCT	GATCTTGAAG
	310	320	330	340	350
5	ACCTAAAACG	CCAAGTACAA	CAACATAAGG	TAGGTGTATC	TTATGTTGCG
	360	370	380	390	400
	TGCTTTCTAC	TAGAAAGCAA	ACTCTGTGTA	TAGTACCTAT	ACACAGTAAC
	410	420	430	440	450
	ACAGATGACA	TGGTTGATGG	GAGAGAATTA	AAACTTAAAG	TCAGCCATAT
10	460	470	480	490	500
	TTTAAAAATT	ATTTTTACCT	AATTGTTTTT	GCAATCTTTG	TTGCCAATGG
	510	520	530	540	550
	CCTTGAATAA	GTCCCCCTCA	AAATTCAGGT	GATTGTATTA	GGAGATGGAA
	560	570	580	590	600
15	TATTTAAGGG	TGAATAATCC	ATCAGGGCTC	CTCCCTTAAG	AATAGGATCA
	610	620	630	640	650
	AGTCCCATAT	AAAAGAGGCT	TCACACAGTG	TTCTCCTATC	TCTTGACCCT
	660	670	680	690	700
	CCACCATGCA	CCACCATGTG	AAAACCTGT	GAAAAGGCCC	TCACCAGATG
20	710	720	730	740	750
	CTAACATCTT	GATCTTGGAT	TTCCCAAAC	CGAGAACTGT	GAAAAAATAA
	760	770	780	790	800
	AGGTACATTC	TTCCTAAATT	ACCTCATCT	CATTTAACA	CACAAAGTGC
	810				
25	ACACATAGCT	G-3'			

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

14. A DNA sequence of the formula:

	5'	10	20	30	40	50
	TTACTGGTGG	AAGAGTTGCC	CCTGCGCCAG	GGAATTCTCA	AACAATTAAA	
35	60	70	80	90	100	
	TGAAACTGGA	GGACCCGTGC	TTGTAAGTGC	TCCCATAAGC	CCAGAAGAGC	
	110	120	130	140	150	
	AAGATAAACT	TGAAAATAAG	CTCAAGCAGA	CAAATCTCCA	GTGGATAAAG	
	160	170	180	190	200	
40	GTTAGACATT	AACCATCTCT	TCCGTCACAT	GTGTTAAATG	TTGCAAGTAT	
	210	220	230	240	250	
	TTGTATGTAT	TTTGTTCCT	GGGTGCTTCA	TTGGTCGGGG	AGGAGGCTGG	
	260	270	280			
45	TATGTGGATT	GTTGTTTTGT	TTTGTTTTTT-3'			

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

15. A DNA sequence of the formula:

	5'	10	20	30	40	50
	AAGCTTTGAT	ACTGTGCTTT	AAGTGTTTAC	CCTTTGGAAA	GAAAATAATT	
	60	70	80	90	100	
5	TTGACAGTGA	TGTAGAAATA	ATTATTTGAT	ATTTATTTC	AAACAAAATT	
	110	120	130	140	150	
	TATATCCAAT	ACTAAACACA	GAATTTTGTA	AAACAATAAG	TGTATAAAGT	
	160	170	180	190	200	
	AAAATGAACA	TTAGGATTAT	TGAGATTATT	GTAGCTAAAA	CTAGTGTTTA	
10	210	220	230	240	250	
	TTCATATAAA	TTATGTTAAT	AAATTGTATT	GTCATTATTG	CATTTTACTT	
	260	270	280	290	300	
	TTTTGAAAAG	TAGTTAATGC	CTGTGTTTCT	ATATGAGTAT	TATATAATTC	
	310	320	330	340	350	
15	AAGAAGATAT	TGGATGAATT	TTTTTTTAA	GTTTAATGTG	TTTCACATCT	
	360	370	380	390	400	
	CTGTTTCTTT	TCTCTGCACC	AAAAGTCACA	TTTTTGTGCC	CTTATGTACC	
	410	420	430	440	450	
	AGGCAGAAAT	TGATCTGCAA	TACATGTGGA	GTCTCCAAGG	GTATATTTAA	
20	460	470	480	490	500	
	ATTTAGTAAT	TTTATTGCTA	ACTGTGAAGT	TAATCTGCAC	TATATGGGTT	
	510	520	530	540	550	
	CTTTTCCCCA	GGAAACTGAA	ATAGCAGTTC	AAGCTAAACA	ACCGGATGTG	
	560	570	580	590	600	
25	GAAGAGATTT	TGTCTAAAGG	GCAGCATTTG	TACAAGGAAA	AACCAGCCAC	
	610	620	630	640	650	
	TCAGCCAGTG	AAGGTAATGA	AGCAACCTCT	AGCAATATCC	ATTACCTCAT	
	660	670	680	690	700	
	AATGGGTAT	GCTTCGCCTG	TTGTACATTT	GCCATTGACG	TGGACTATTT	
30	710	720	730	740	750	
	ATAATCAGTG	AAATAACTTG	TAAGGAAATA	CTGGCCATAC	TGTAATAGCA	
	760	770	780	790	800	
	GAGGCAAAGC	TGTCTTTTGT	ATCAGCATAT	CCTATTTATA	TATTGTGATC	
	810	820	830	840		
35	TTAAGGCTAT	TAACGAGTCA	TTGCTTTAAA	GGACTCATTT	CTGTC-3'	

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

40 16. A DNA sequence of the formula:

	5'	103	113	123	133	143
	CCCATCTTGT	TTTGCCTTTG	TTTTTTCTTG	AATAAAAAAA	AAATAAGTAA	
45	153	163	173	183	193	
	AATTTATTTT	CCTGGCAAGG	TCTGAAAAC	TTTGTTTTCT	TTACCACTTC	

50

55



	203	213	223	233	243
	CACAATGTAT	ATGATTGTTA	CTGAGAAGGC	TTATTTAACT	TAAGTTACTT
	253	263	273	283	293
6	GTCCAGGCAT	GAGAATGAGC	AAAATCGTTT	TTTAAAAAAT	TGTTAAATGT
	303	313	323	333	343
	ATATTAATGA	AAAGGTTGAA	TCTTTTCATT	TTCTACCATG	TATTGCTAAA
	353	363	373	383	393
	CAAAGTATCC	ACATTGTTAG	AAAAAGATAT	ATAATGTCAT	GAATAAGAGT
10	403	413	423	433	443
	TTGGCTCAAA	TTGTTACTCT	TCAATTAAAT	TTGACTTATT	GTTATTGAAA
	453	463	473	483	493
	TTGGCTCTTT	AGCTTGTGTT	TCTAATTTTT	CTTTTTCTTC	TTTTTTCCTT
	503	513	523	533	543
15	TTTGCAAAAA	CCCAAAATAT	TTTAGCTCCT	ACTCAGACTG	TTACTCTGGT
	553	563	573	583	593
	GACACAACCT	GTGGTTACTA	AGGAACTGC	CATCTCCAAA	CTAGAAATGC
	603	613	623	633	643
	CATCTTCCTT	GATGTTGGAG	GTACCTGCTC	TGGCAGATTT	CAACCGGGCT
20	653	663	673	683	693
	TGGACAGAAC	TTACCGACTG	GCTTTCCTCTG	CTTGATCAAG	TTATAAAATC
	703	713	723	733	743
	ACAGAGGGTG	ATGGTGGGTG	ACCTTGAGGA	TATCAACGAG	ATGATCATCA
	753	763	773	783	793
25	AGCAGAAGGT	ATGAGAAAAA	ATGATAAAAG	TTGGCAGAAG	TTTTTCTTTA
	803	813	823	833	843
	AAATGAAGAT	TTTCCACCAA	TCACTTTACT	CTCCTAGACC	ATTTCCCACC
	853	863	873	883	893
	AGTTCTTAGG	CAACTGTTTC	TCTCTCAGCA	AACACATTAC	TCTCACTATT
30	903	913	923	933	943
	CAGCCTAAGT	ATAATCAGGT	ATAAATTAAT	GCAAATAACA	AAAGTAGCCA
	953	963	973	983	993
	TACATTAAAA	AGGAAAATAT	ACAAAAAATA	AAAAAAAAAA	AAGCCAGAAA
	1003	1013			
35	CCTACAGAAT	AGTGCTCTAG	TAATTAC 3'		

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

40 17. A DNA sequence of the formula:

	5'	10	20	30	40	50
	ATCTCTATCA	TTAGAGATCT	GAATATGAAA	TACTTGTCAA	AGTGAATGAA	
45	60	70	80	90	100	
	AATTTNNTAA	ATTATGTATG	GTTAACATCT	TTAAATTGCT	TATTTTAAAA	
	110	120	130	140	150	
	TTGCCATGTT	TGTGTCCCAG	TTTGCAATTA	CAAATAGTTT	GAGAACTATG	
	160	170	180	190	200	
50	TTGGAAAAAA	AAATAACAAT	TTTATTCTTC	TTTCTCCAGG	CTAGAAGAAC	

55

	210	220	230	240	250
	AAAAGAATAT	CTTGTCAGAA	TTTCAAAGAG	ATTTAAATGA	ATTTGTTTTA
	260	270	280	290	300
5	TGGTTGGAGG	AAGCAGATAA	CATTGCTAGT	ATCCCACTTG	AACCTGGAAA
	310	320	330	340	350
	AGAGCAGCAA	CTAAAAGAAA	AGCTTGAGCA	AGTCAAGGTA	ATTTTATTTT
	360	370	380	390	400
	CTCAAATCCC	CCAGGGCCTG	CTTGCCATAAA	GAAGTATATG	AATCTATTTT
10	410	420	430	440	450
	TTAATTCAAT	CATTGGTTTT	CTGCCCATTG	GGTTATTCAT	AGTTCCTTGC
	460	470	480	490	500
	TAAAGTGTTT	TTCTCACAAAC	TTTATTTCTT	CTTAACCCTG	CAGTTCTGAA
	510	520	530	540	550
15	CCAGTGACAA	TAAGAACATA	TGTATATATG	TGTGTGTGTG	TATTTATATA
	560	570	580	590	600
	TACACACACA	CATATTGCAT	CTATACATCT	ACACATATAG	ATGTATAGAT
	610	620	630	640	650
	TCAATATGTC	TAAAAATGTA	TATAATTAC	AGTTTTTATC	TTTGATTGGA
20	660	670	680		
	ATATTTAAGG	GACTGAGACT	CACACTCATA	TACTTTT-3'	

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

30

35

40

45

50

55

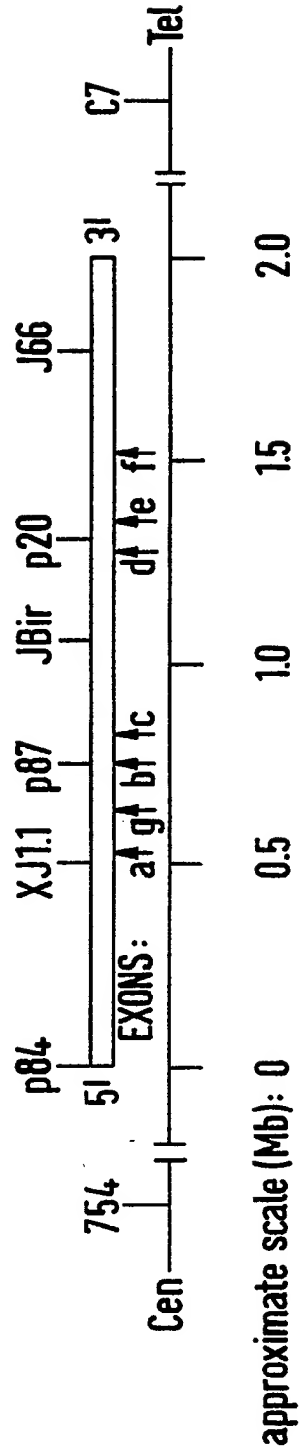
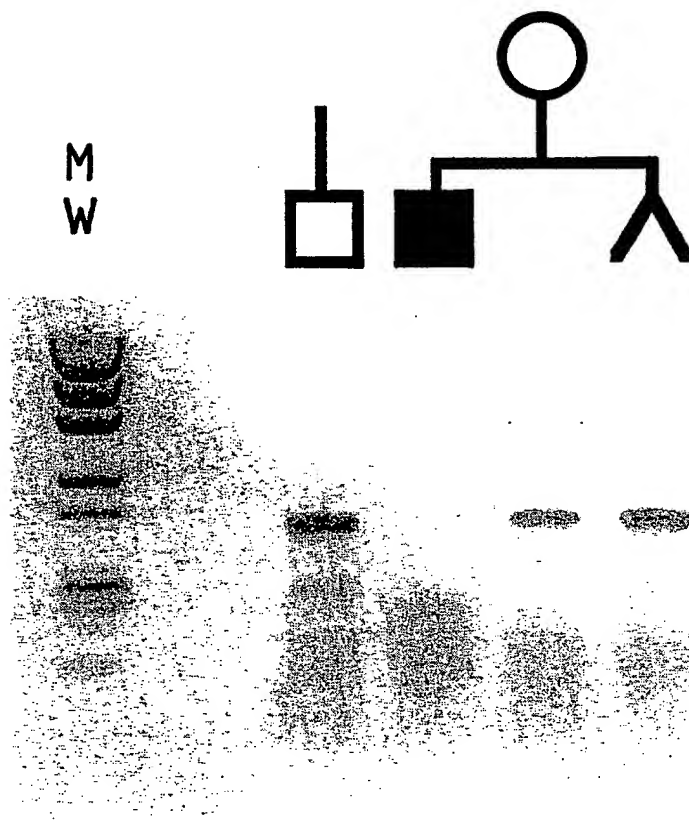


FIG.1



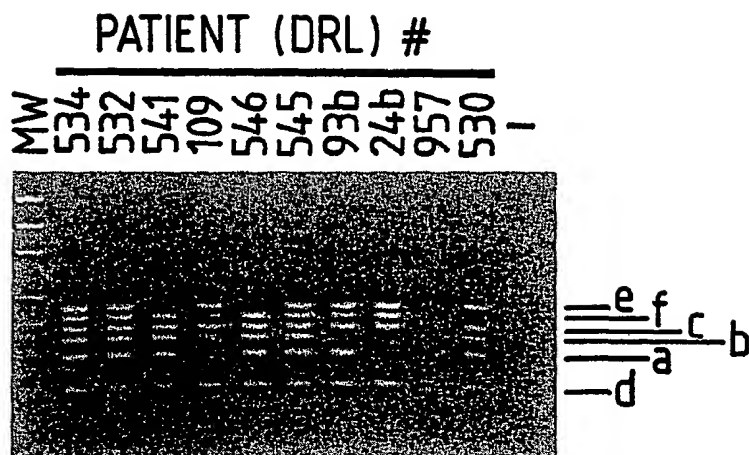


FIG. 3A

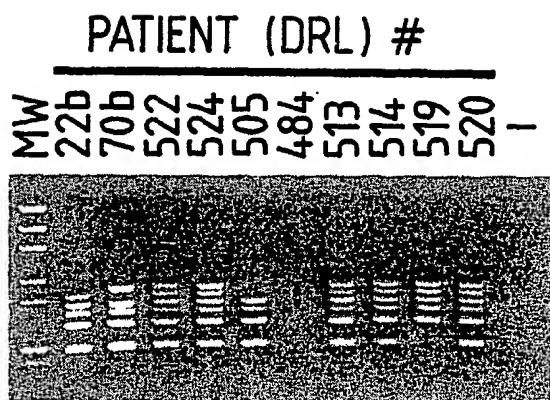
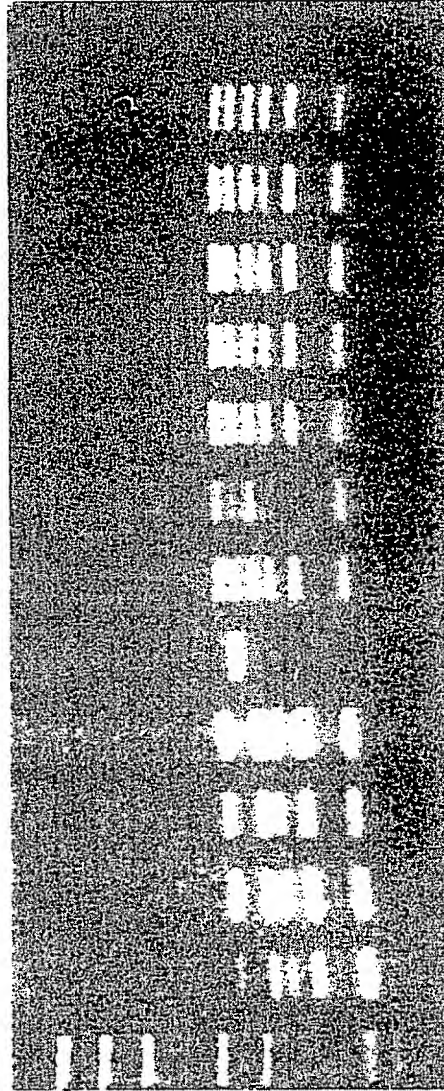
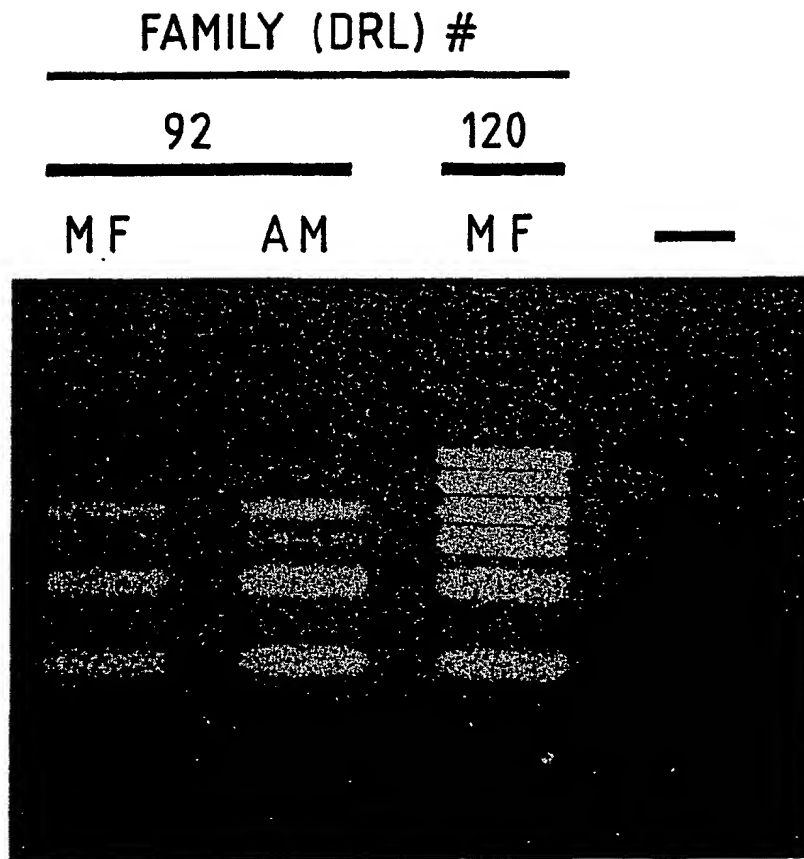


FIG. 3B

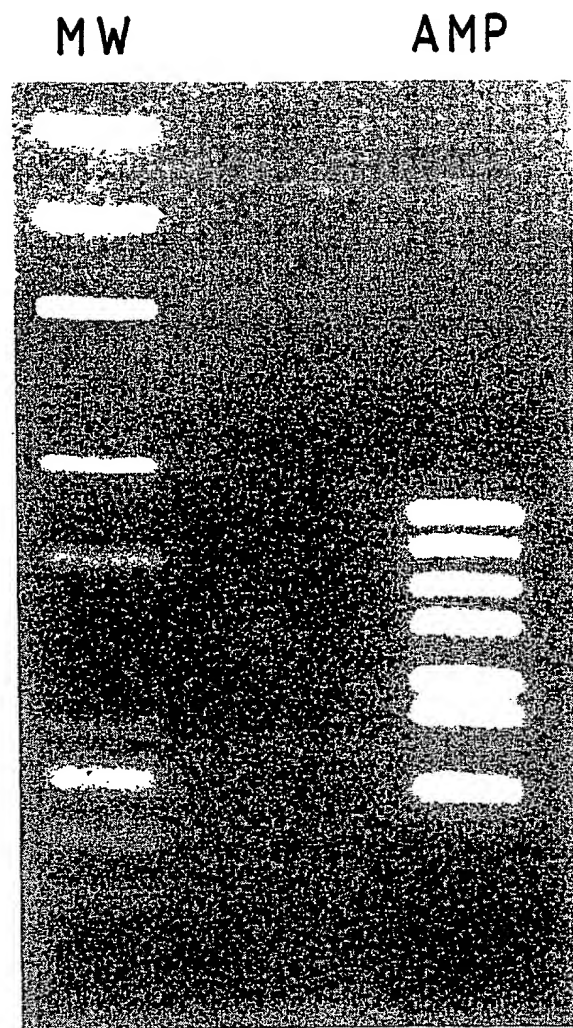
FAMILY (DRL) #					
521	531	43C	483	485	469
A M	M F	A M	M F	A M	A M
A M	A M	M F	A M	M F	M F
M W	M F	M F	M F	M F	M F



**FIG. 4**



**FIG.5**



**FIG. 6**